Optimization of antibody-conjugated magnetic nanoparticles for target preconcentration and immunoassays

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ABSTRACT

Biosensors based on antibody recognition have a wide range of monitoring applications that apply to clinical, environmental, homeland security, and food problems. In an effort to improve the limit of detection of the Naval Research Laboratory (NRL) Array Biosensor, magnetic nanoparticles (MNPs) were designed and tested using a fluorescence-based array biosensor. The MNPs were coated with the fluorescently labeled protein, AlexaFluor647–chicken IgG (Alexa647–chick IgG). Antibody-labeled MNPs (Alexa647–chick–MNPs) were used to preconcentrate the target via magnetic separation and as the tracer to demonstrate binding to slides modified with anti-chicken IgG as a capture agent. A full optimization study of the antibody-modified MNPs and their use in the biosensor was performed. This investigation looked at the Alexa647–chick–MNP composition, MNP surface modifications, target preconcentration conditions, and the effect that magnetic extraction has on the Alexa647–chick–MNP binding with the array surface. The results demonstrate the impact of magnetic extraction using the MNPs labeled with fluorescent proteins both for target preconcentration and for subsequent integration into immunoassays performed under flow conditions for enhanced signal generation.

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Biosensors are under development for target screening in clinical, environmental, water, and food samples [1–4]. An essential component of these systems is the recognition elements, often antibodies, for selective identification of target analytes. Antibodies have demonstrated high binding affinities with extraordinary specificity for target molecules even in complex sample matrices and with low target concentrations [5]. The Array Biosensor developed at the Naval Research Laboratory (NRL),[2] which typically performs multiplexed immunoassays, has been used successfully for the detection of a variety of proteins, molecules, viruses, and bacteria in complex sample matrices [6,7]. The two-dimensional nature of the sensing surface facilitates simultaneous analysis of multiple samples for multiple analytes. The immunoassays developed to date are rapid (15–25 min) and automated, with little or no sample pretreatment prior to analysis [8]. Limits of detection (LOD) obtained with the NRL Array Biosensor are comparable to other rapid biosensor technologies and enzyme-linked immunosorbent assays (ELISAs). However, the NRL system falls short of the LODs desired for some targets, particularly bacterial species, compared with those obtained by the more time-consuming and complex "gold standard" methodologies such as cell culture and polymerase chain reaction (PCR). To overcome this limitation, one approach would be to include a target preconcentration step prior to the immunoassay. However, to keep the detection method practical, any sample treatment steps must be simple to perform, add minimal time to the analysis, and improve the overall assay results.

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[2] Abbreviations used: NRL, Naval Research Laboratory; LOD, limit of detection; ELISA, enzyme-linked immunosorbent assay; PCR, polymerase chain reaction; MP, magnetic particle; HSA, human serum albumin; MNP, magnetic nanoparticle; MATEFF, magnetically assisted transport evanescent field fluoroimmunoassay; Alexa647–chick–MNPs, MNPs functionalized with fluorescently labeled target chicken IgG with AlexaFluor647; MTS, 3-mercaptopropyl trimethoxy silane; GMBS, N-(γ-maleimidobutyryloxy) succinimide ester; TEOS, tetraethylorthosilicate; Mes, 2-(N-morpholino) ethane sulfonic acid; PDMS, poly(dimethyl) siloxane; carboxyl–silane, carboxyethylsilanetriol sodium salt; EDC, 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride; NHS, N-hydroxysuccinimide; Rb–anti-chick IgG, rabbit anti-chicken IgY; PBS, phosphate-buffered saline; UV–Vis, ultraviolet–visible; PEG, polyethylene glycol; BSA, bovine serum albumin; PBST, PBS + 0.05% Tween; PBSCD, PBS/0.1% casein/0.05% deoxycholic acid; CCD, charge-coupled device; DOC, deoxycholic acid.

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14. ABSTRACT

Biosensors based on antibody recognition have a wide range of monitoring applications that apply to clinical environmental, homeland security, and food problems. In an effort to improve the limit of detection of the Naval Research Laboratory (NRL) Array Biosensor, magnetic nanoparticles (MNPs) were designed and tested using a fluorescence-based array biosensor. The MNPs were coated with the fluorescently labeled protein, AlexaFluor647?chicken IgG (Alexa647?chick IgG). Antibody-labeled MNPs (Alexa647? chick?MNPs) were used to preconcentrate the target via magnetic separation and as the tracer to demonstrate binding to slides modified with anti-chicken IgG as a capture agent. A full optimization study of the antibody-modified MNPs and their use in the biosensor was performed. This investigation looked at the Alexa647?chick?MNP composition, MNP surface modifications, target preconcentration conditions and the effect that magnetic extraction has on the Alexa647?chick?MNP binding with the array surface. The results demonstrate the impact of magnetic extraction using the MNPs labeled with fluorescent proteins both for target preconcentration and for subsequent integration into immunoassays performed under flow conditions for enhanced signal generation.

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Immunomagnetic separation (IMS) is one preconcentration technique that is commonly used prior to detection for sample preparation and cleanup. Magnetic particles (MPs) are becoming increasingly popular for automated separations [9,10]. These magnetic materials are easily manipulated using magnetic fields and are removed from solutions in a matter of minutes. With surface modification, MPs have been labeled with a variety of biological molecules that have the ability to scavenge for targets of interest and separate them from complex biological media, potentially improving the LOD of subsequent analysis techniques. Commercially available MPs are typically 0.5 to 2 μm in diameter and come with a variety of chemically active surfaces that can be used to functionalize the particle with the desired capture agent, offering a large surface area for target capture.

Common formats for quantification of targets collected by MPs are typically independent of the particles themselves. Such methods include culture, flow cytometry analysis [11], PCR coupled with hybridization [12], electrochemical measurements [13,14], and ELISAs [15–17]. When fluorescence species are added, quantification of the resulting fluorescent immunomagnetic–target complex is normally achieved using devices such as a spectrometer [18,19], a flow cytometer [11,20], or a fluorescence microscope [21,22]. Increasingly, researchers are using the properties of the MPs themselves to determine the presence of the bound target [23,24] with devices such as giant magnetoresistive (GMR) sensors [25,26], the superconducting quantum interference device (SQUID) [27], and the magnetic permeability-based assay [28]. Interestingly, Colombo and coworkers [29] recently used the proton T_2 relaxation time of water molecules surrounding human serum albumin (HSA)-modified magnetic nanoparticles (MNPs) as a sensor for anti-HSA detection.

Advances in microfluidics and integrated technologies have resulted in the use of MPs coupled with planar surfaces [15,16,24–26]. Wellman and Sepaniak [30] demonstrated that magnetic beads functionalized with a fluorescence antibody complex could be transported, using an external magnetic field, into the region of an evanescent field for detection, a technique referred to as magnetically assisted transport evanescent field fluoroimmunoassay (MATEFF). Morozov and Morozova [31] investigated a number of methods for interacting antibody-labeled MPs with protein microarrays, including a magnetic brushing technique, magnetic scanning, and a push/pull method that used a magnet below the substrate to concentrate the beads on the surface and a second magnet above the substrate to remove weakly bound or nonspecifically bound MPs [31]. They recently extended their studies to look more closely at force differentiation and shear stress under flow [32]. These studies use complicated schemes to facilitate MP interaction with the surface, in large part due to the relatively large sizes of the commercial MPs used. The binding of large antigen–antibody–MP complexes to an antibody immobilized on a sensor surface is subject to shear and subsequent dissociation from the surface in the flow conditions normally used in such immunoassays [32]. One way to address this problem would be to decrease the size of the MPs used. Nano-sized magnetite particles enveloped in lipid membranes, produced by bacteria, have been used in a number of studies [33]. Modifiable iron oxide-based MNPs have also been synthesized with a well-defined size and shape [34,35]. MNPs are being used in ELISAs [15–17], lateral flow immunoassays [36], and a magnetic force microscopy bioassay for biotin–streptavidin [37] and IgG detection [38]. Although Morozov and Morozova [31,32] have studied micron-sized MPs interacting with protein microarrays, to date there are limited studies that use nano-sized MPs to facilitate both target concentration and signaling events for immunoassays [36–38].

In this study, fluorescently tagged antibodies attached to MNPs were employed in a simple target preconcentration step. The extracted target–antibody–MNPs were introduced directly to the Array Biosensor under flow conditions to initiate signal transduction, and the effect of the target preconcentration and nanoparticle-based fluorescence signal generation was evaluated. This method was used to improve the overall LOD of the Array Biosensor (Scheme 1). Unlike the previously mentioned MATEFFs, these MPs are not used simply to localize the target to the evanescent field sensing surface but rather to simultaneously perform both a target concentration and a signaling function on the microarray. For optimization purposes, a simple direct binding assay was investigated. MNPs were functionalized with the fluorescently labeled target chicken IgG with AlexaFluor647 (Alexa647–chick–MNPs). The sensor surface was patterned with rabbit–anti-chicken IgG. The assay was used to evaluate the surface composition of the modified MNPs prepared under a variety of conjugation conditions, the extraction time for preconcentration experiments, the sample concentration achieved by magnetic extraction, and the effect of magnetic extraction on the MNP on particle aggregation and binding. The final signals were produced as the result of the binding of the MNPs to the slide surface.

Materials and methods

Materials

Unless otherwise specified, chemicals were of reagent grade and used as received. All chemicals, including 3-mercaptopropyl trimethoxy silane (MTS), N-(γ-maleimidobutyryloxy) succinimide ester (GMBS), tetraethylorthosilicate (TEOS), and 2-(N-morpholino) ethane sulfonic acid (Mes), were purchased from Sigma–Aldrich (St. Louis, MO, USA) unless otherwise noted. Poly(dimethyl) siloxane (PDMS), used for making the assay flow cells, was obtained from Nusil Silicone Technology (Carpinteria, CA, USA). Borosilicate glass slides from Daigger (Vernon Hills, IL, USA) were used in all of the
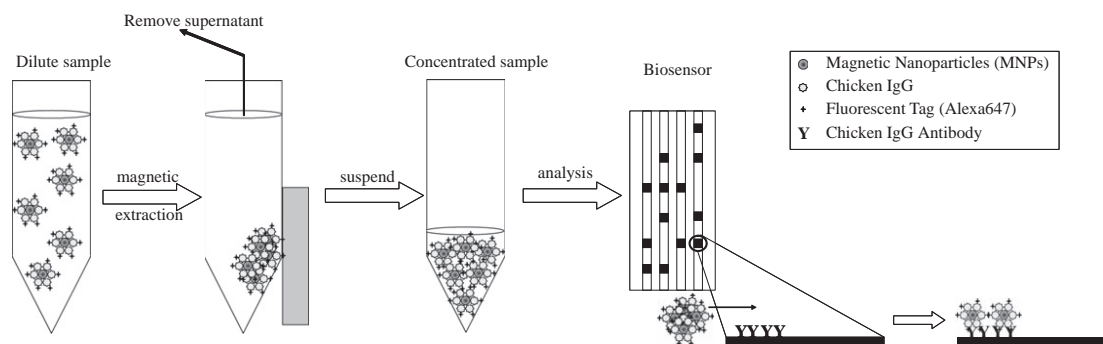
Scheme 1. Magnetic extraction, sample concentration, and analysis. The MNPs modified with fluorescently tagged antibody are extracted from the large volume sample, the extracted MNPs are resuspended in 0.2 ml of buffer, and analysis is performed using the Array Biosensor with the MNPs functioning as the tracer.

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assays described. Carboxyethylsilanetriol sodium salt (carboxyl–silane) was purchased from Gelest (Morrisville, PA, USA). 1-Ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC), N-hydroxysuccinimide (NHS), and NeutrAvidin were purchased from Pierce Biotechnology (Rockford, IL, USA). Ammonium hydroxide was obtained from Fisher. The biotin-SP-conjugated rabbit anti-chicken IgY (Rb–anti-chick IgG) and chicken IgY (chick IgG) were purchased from Jackson ImmunoResearch (West Grove, PA, USA). Note that IgY is the original designation for the IgG-like protein found in both serum and egg yolk; therefore, IgG is used throughout this article. Fluorescent labeling of the chicken IgG was achieved using succinimide ester-functionalized Alexa647 purchased from Molecular Probes (Eugene, OR, USA).

MNP synthesis

Iron oxide MNPs were synthesized by coprecipitating iron salts. Using a mechanical stirrer, a 155-ml solution of ammonia hydroxide (2.5%) and iron chloride was mixed at 350 rpm for 10 min as described previously [39]. The iron salt solution was ferric chloride hexahydrate (0.5 M), ferrous chloride tetrahydrate (0.25 M), and HCl (0.33 M) to a final volume of 100 ml. The MNPs generated were washed with 3-ml aliquots of water three times and with ethanol once. The MNPs were dispersed in a 3-ml ethanol solution that contained approximately 1.2% ammonium hydroxide at a final concentration of approximately 7.5 mg/ml.

The MNPs were coated with silica by adding 200 µl of TEOS. The hydrolysis process was conducted while sonicating for 90 min. Another aliquot of TEOS (10 µl) was added, and sonication was continued for an additional 90 min. The sample was again washed with 3-ml aliquots of ethanol three times. An 80-µl aliquot of the carboxyl–silane as a sodium salt was added to 1 ml of 10 mg/ml silica-coated MNPs in 10 mM phosphate-buffered saline (PBS, pH 7.4) and continuously mixed for 4 h. Finally, the particles were washed three times with 10 mM PBS and stored at room temperature until used.

Dye labeling of chick IgG

AlexaFluor labeling of the chick IgG prior to attachment to the MNPs was carried out according to the procedure of Anderson and Nerurkar [40]. Labeled antibodies were separated from unincorporated dye using size exclusion chromatography (BioGel P10). Protein-to-dye ratios were determined using ultraviolet–visible (UV–Vis) spectroscopy.

Optimized MNP labeling with Alexa647–chick IgG

The overall goal of this study was to optimize protein immobilization onto the surface of the MNPs with respect to its subsequent imaging on the NRL Array Biosensor. This section describes the optimized conditions for the best MNPs for the dual use of concentrating the analyte and as the tracer for the array. The investigation described here was the research involved to arrive at this optimized system for the described purpose of using MNPs for the improvement of the LOD for the NRL Array Biosensor. A number of parameters were investigated, as highlighted in Results and discussion. The following protocol represents the final optimized procedure only and not all of the prerequisite investigations required in arriving at this optimized procedure. A 250-µl solution of 4 mg/ml carboxyl-modified MNPs was washed three times with 250-µl aliquots of 0.5 mM Mes buffer (pH 5.0). Modification of the chicken IgG was carried out by adding 50 µl of a 20-mg/ml EDC solution to the washed particles and incubated for 15 min. Next, 100 µg of Alexa647–chick IgG with a 1:5 molar equivalent of an amine–PEG (polyethylene glycol, 5000 Da) was added to the activated MNPs. The solution was incubated for 2 h with vortexing every 15 to 30 min. The MNPs were magnetically extracted and washed three times with 500-µl aliquots of 10 mM PBS buffer. After the third wash, the Alexa647–chick–MNP complex was resuspended in 500 µl of 30 mM hydroxylamine with 1% bovine serum albumin (BSA) in 10 mM PBS buffer (pH 7.4) and incubated for 30 min. Finally, the Alexa647–chick–MNPs were washed three times and resuspended in 500-µl aliquots of 10 mM PBS with 0.05% Tween 20 and 0.1% BSA at pH 7.4. The final concentration of the MNPs was 2 mg/ml, and the samples were stored at 4 °C until used. For use, the Alexa647–chick–MNPs were diluted in buffer for final concentrations ranging from 0.01 to 0.4 mg/ml.

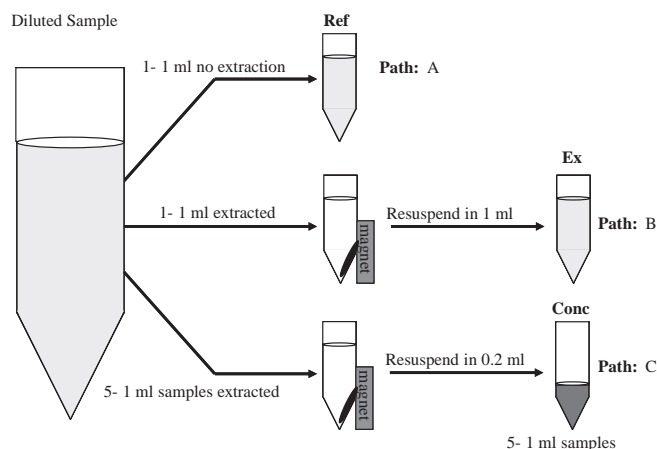### Slide preparation, MNP extraction, and immunoassay

Microscope slides, used as waveguides, were cleaned by immersion in 10% (w/v) KOH in 2-propanol for 30 min at room temperature, followed by rinsing with 18 mΩ Milli-Q water and drying with a nitrogen stream. The slides were immediately immersed in a toluene solution containing 2% MTS for 1 h under nitrogen. The silanized slides were then rinsed with toluene, dried with nitrogen, and immediately immersed in 1 mM GMBS in absolute ethanol for 30 min at room temperature. The slides were rinsed with water and incubated in 25 µg/ml NeutrAvidin in PBS overnight at 4 °C before being washed in PBS (pH 7.4). Slides were either used immediately for patterning or stored in PBS at 4 °C until required. Patterning of the biotinylated Rb–anti-chick IgG (10 µg/ml) in PBS + 0.05% Tween (PBST) was carried out using a 6-channel patterning PDMS flow cell clamped onto the NeutrAvidin-functionalized slide surface and injecting the biotinylated capture antibody into 4 or 5 of the channels [6–8]. Biotinylated goat anti-mouse IgG (10 µg/ml in PBST) was introduced into the remaining channels for use as a negative control. The slides were then incubated overnight at 4 °C. After the channels were rinsed with 1 ml of PBST, the slide was removed from the PDMS patterning template and placed in PBS blocking solution containing 1% casein. After approximately 1 h, the slides were rinsed with 18 mΩ Milli-Q water and assembled in a 6- or 12-channel assay PDMS flow cell, with the flow channels orientated perpendicular to the stripes of immobilized biotinylated antibodies. Each channel was hooked up to an ISMATEC multichannel pump (Cole–Parmer Instruments, Vernon Hills, IL, USA) at one end (outlet), and syringe barrels (1 ml) were then attached at the opposite end (inlet), ready for the immunoassay.

The Alexa647–chick–MNPs were first sonicated and vortexed briefly to suspend the sample. The Alexa647–chick–MNPs were directly diluted in 1 ml of the PBS/0.1% casein/0.05% deoxycholic acid (PBSCD) buffer (pH 7.4) for direct immunoassays or prepared for extraction studies. A typical extraction procedure involved diluting 200 µl of the stock Alexa647–chick–MNPs in 10 ml of PBSCD. The samples were prepared for the assay as described in detail in Scheme 2 (see description below). The samples that were treated by paths A and B were not concentrated, and the samples that follow path C were concentrated 5-fold prior to introduction into the Array Biosensor.

The MNPs labeled with Alexa647–chick IgG, prepared as described above and sonicated for 5 min, were applied to each channel (0.8 ml) at a flow rate of 0.1 ml/min. The channels were then washed with 1 ml of PBSCD at 0.25 ml/min. The PDMS flow cell was removed, and the slide was washed with 18 mΩ Milli-Q water, dried with nitrogen, and imaged on the Array Biosensor.

Immunoassay array imaging and analysis

The slides were imaged using a Peltier-cooled charge-coupled device (CCD) camera as described previously [6–8]. Briefly,

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Scheme 2. Sample extraction and concentration of the MNPs. Three sample classifications exist: Ref obtained following path A, Ex obtained following path B, and Conc obtained following path C. The Ref sample is a sample taken directly from the diluted sample with no magnetic extraction performed (no concentration). Both the Ex and Conc samples are magnetically extracted. However, after magnetic extraction, the supernatant is removed from the Ex sample and the sample is suspended in the volume of 1 ml (resulting in no concentration). For the Conc sample, the sample is suspended in a smaller volume of 0.2 ml after extraction and removal of the supernatant. This caused a 5 times concentration of the extracted MNPs.

evanescent wave excitation of the surface-bound fluorescent species was achieved using a 635-nm, 12-mW diode laser (LaserMax, Rochester, NY, USA). Light was launched into one end of the slide at an appropriate angle through a 1-cm focal length lens equipped with a line generator to generate evanescent wave excitation. The fluorescence emission was monitored at right angles to the planar surface. A two-dimensional graded index of refraction (GRIN) lens array (Nippon Sheet Glass, Somerset, NJ, USA) was used to image the fluorescent pattern onto the Peltier-cooled CCD camera (Spectra Source, Teleris, Westlake Village, CA, USA) [6–8]. Long-pass (Schott 0G-0665, Schott Glass, Duryea, PA, USA) and bandpass (Corion S40-670-S, Franklin, MA, USA) filters were mounted on the device scaffolding to eliminate excitation and scattered light prior to CCD imaging.

Data were acquired in the form of digital image files in Flexible Image Transport System (FITS) format. To analyze the images, a custom software application was written in LabWindows/CVI (National Instruments). The program creates a mask consisting of data squares (enclosing the areas where the capture antibody is patterned) and background rectangles that are located on either side of each data square. The average background value is subtracted from the average data square value, and the net intensity value is calculated and imported into a Microsoft Excel file for data analysis.

Results and discussion

MPs on the array biosensor

In an effort to improve this biosensor's LOD while maintaining a rapid analysis time, the concept of immunomagnetic concentration of the target coupled with simultaneous fluorescent labeling was investigated (see Scheme 1). In general, magnetic extraction was performed by using a permanent magnet, the supernatant was removed, and the extracted sample was suspended in 0.2 ml of buffer. This concentrated sample was then introduced to the Array Biosensor under flow conditions, and the array was analyzed using a CCD camera. This method was employed while operating the biosensor with the MNPs as the tracer. In addition, the effect that

magnetic extraction has on the MNP array binding event was also explored. For these studies, the optimization, demonstration of sample concentration, and operation of the array with the MNP tracers were performed using a direct binding assay. The assay used Alexa647–chick–MNPs and slides patterned with Rb–anti-chick IgG slide surfaces.

For the initial investigations with micron-sized MPs (see Supplementary Fig. 1 in supplementary material), the samples were not extracted prior to introducing the samples to the Array Biosensor. The microparticles were internally labeled with Alexa647 and functionalized with chicken IgG [41]. In these studies, MPs modified with chicken IgG in various buffer conditions, surfactants, and blocking proteins were investigated. The MPs demonstrated successful binding under static conditions with buffer containing casein (1%) and deoxycholic acid (DOC, 0.05%) to minimize nonspecific particle array surface binding. However, the direct binding assay performed poorly under flow conditions, resulting in low signal intensities. This poor assay performance was probably due to the large diameter of the magnetic beads and the shear force at the surface under flow conditions.

To address the size issue, smaller nano-sized MNPs synthesized in-house were investigated. Iron oxide core MNPs, coated with silica, were functionalized with carboxyl–silane, followed by the attachment of chicken IgG via EDC coupling chemistry. Transmission electron microscope (TEM) and scanning tunneling microscope (STM) images suggest that the nanoparticles are spherical, silica-coated MNPs with sizes from 30 to 120 nm, although the majority exhibited an approximately 65-nm diameter (Supplementary Fig. 2). To generate a signal in the evanescent field of the NRL Array Biosensor, the chicken IgG attached to the MNPs was also labeled with Alexa647 dye. Typical ratios were kept between 2 and 4 dye molecules per chicken IgG, as determined by UV–Vis spectroscopy, to ensure that free lysines were available for coupling to the MNP surface.

A number of factors that could affect the MNP performance were investigated, particularly when these silica-based materials are used with a surface such as the glass slides. As a starting point, the EDC-activated MNPs were initially exposed to 350 μg of purified Alexa647–chick IgG. The Alexa647–chick IgG MNPs were then diluted 50, 20, or 5 μl in 1 ml of the running buffer PBSCD (PBS/0.1% casein/0.1% DOC) for assay studies. A CCD image of these initial assays is shown in Fig. 1. The first four lanes of the array were modified with rabbit–anti-chicken for the target capture, and the fifth lane was modified with goat-anti-mouse to serve as the control.

Strong signals were found in the regions of the slide functionalized with Rb–anti-chick IgG. The signal intensity was concentration dependent. No signal was observed in the control lane, demonstrating the specificity of the interaction. More important, these initial experiments illustrate that these antigen-coated MNPs, unlike their larger counterparts (see Supplementary Fig. 1), bind to the surface under flow conditions. However, the nonuniform/speckled fluorescence signal observed in Fig. 1 suggests that aggregation of the MNPs was problematic. Aggregation
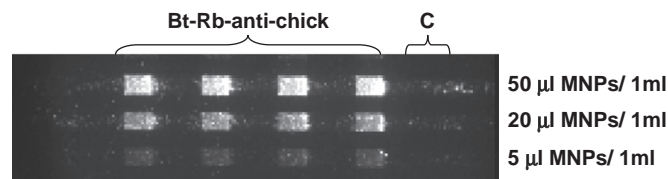

Fig.1. Assay under flow conditions using the Alexa647–chick–MNPs at varying concentrations at a flow rate of 0.1 ml/min. The first four lanes of the array were modified with biotinylated (Bt) rabbit–anti-chicken IgG, and the fifth lane was the control lane of the array (labeled C) and was modified with goat–anti-mouse IgG.

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causes the nanoparticles to behave like larger particles rather than monodispersed nanoparticles, and it was possible that even larger aggregates were dissociated from the surface by sheer forces. This investigation demonstrated that the nano-sized particles showed promise as a tracer under flow conditions for the Array Biosensor. However, further experimentation to optimize the quality of the MNPs acting as the tracer was required.

Optimization of MNPs as tracer for the array

To improve the performance of these MNPs with the biosensor on the array slide, this aggregation issue was examined. The features studied to limit aggregation include the composition of carboxyl groups present in the MNP modification procedure, the assay buffer conditions, and the amount of Alexa647–chick IgG used by the MNPs and the EDC attachment method in the protein modification reaction. These parameters were investigated both to improve the compatibility of the particles with the array surface and to prevent particle–particle interactions.

The proportion of carboxyl silane present in the surface modification reaction for the MNPs was varied to change the density of charged groups and protein binding sites on the surface. The silica-coated MNP surface was exposed to silane solutions containing 100%, 75%, 50%, or 25% carboxyl groups, with the remainder of the solution consisting of silane containing an EDC-unreactive phosphonate group. These silanes were selected because they maintain a negative charge, and a negatively charged surface is necessary to reduce the aggregation observed by these silica-based nanoparticles. The carboxyl percentages represent the percentages of carboxyl groups present in the solution exposed to the MNPs and do not necessarily equate to the percentages of carboxyl groups present on the final MNP surface. The amount of protein immobilized to the MNPs was either 350 or 100 μg of Alexa647–chick IgG in the presence of EDC-activated MNPs. The Alexa647–chick–MNPs in 0.8 ml (150 μl in 1 ml PBS/0.1% casein/0.05% DOC) were passed over an antibody-functionalized surface at a flow rate of 0.1 ml/min. The resulting bar graph (Fig. 2A) shows the relative intensity of the captured Alexa647–chick–MNPs. The black and gray bars represent the MNPs fabricated using either 350 or 100 μg of Alexa647–chick IgG, respectively. For the 100-μg samples, only 100 and 25% carboxyl were used; these two amounts confirmed the trend based on a more complete range tested using MNPs modified with 350 μg of antigen. The concentration of carboxyl groups on the particle surface was more important for enhancing the use of the particles as a tracer than the concentration of protein used in the coupling reaction. Higher concentrations of carboxyl groups increased the amount of protein bound.

However, in the case of the MNPs exposed to 350 μg of the Alexa647–chick IgG, decreasing the protein concentration on the surface by decreasing the carboxyl concentration did not decrease the speckled nature of the fluorescence signal observed in the CCD images (data not shown). This suggested that aggregation was still an issue for these samples. The speckled nature of the fluorescence signal caused a much greater standard deviation of the intensities within and between spots. This speckled nature caused poor LOD results from the assay and concomitant reproducibility concerns. Therefore, the 100% carboxyl-treated MNPs were used for the remainder of these optimization studies because they produced the greatest amount of absolute fluorescence signal.

Simultaneously, the impact of the assay buffer and the amount of protein used in the MNP immobilization reaction on particle–particle aggregation were evaluated to reduce nonspecific interactions of the particles with the array surface and to promote selective binding of the Alexa647–chick–MNPs with the array surface (Supplementary Fig. 3). The buffer affects nonspecific binding of the nanoparticles due to electrostatic and physical interactions with the array surface as well as with other particles in solution. Supplementary Figs. 3A and 3B show MNPs reacted with 350 μg of Alexa647–chick IgG with PBS/0.1% casein/0.1% Tween 20 and PBS/0.1% casein/0.1% DOC, respectively. The first four lanes of the array were modified with rabbit–anti-chicken for the target capture, and the fifth lane was modified with goat–anti-mouse to serve as the control. Changing the surfactant in the buffer had little effect on eliminating the speckled fluorescence signal observed in the CCD images.

Similarly, the amount of protein in the immobilization reaction was optimized to reduce nonspecific particle–particle solution interaction due to electrostatic and physical interactions. When the amount of Alexa647–chick IgG was reduced from 350 to 100 μg (cf. Supplementary Figs. 3A and 3C) and both samples were analyzed using the PBS/casein/DOC buffer, the resulting fluorescence signal was much more uniform in intensity within the individual data points. This result suggests that less aggregation was observed with the lower concentration of Alexa647–chick used in the protein attachment procedure. Other buffer types were used with the 100 μg Alexa647–chick IgG prepared samples (data not shown), but PBS/casein/DOC provided the highest quality performance of the Alexa647–chick–MNPs on the array.

The next step in the optimization of the MNPs for the array involves the protein attachment reaction procedure. Here the effect of the EDC reaction conditions used to attach 100 μg of Alexa647–chick IgG on the surface of 100% carboxyl MNPs was investigated. In the studies mentioned above, the EDC was not removed from the MNP solution prior to the addition of the Alexa647–chick IgG (EDC method I). Because the protein chicken IgG contains both carboxyl and amine groups, it was possible that multilayers of Alexa647–chick IgG could form on the surface of the MNPs. Multilayers could encourage increased aggregation, causing
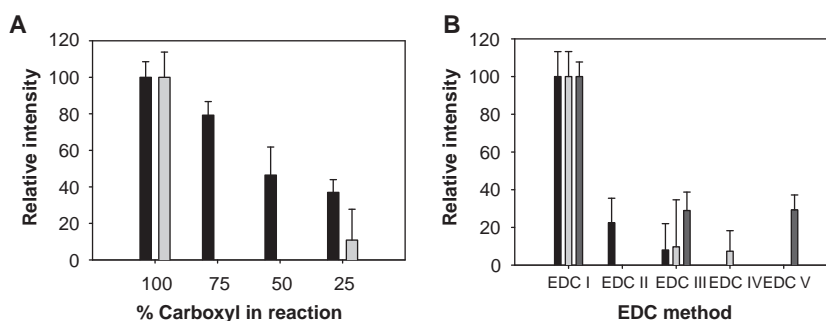

Fig.2. Optimization of the MNP surface treatment. (A) Effect of the percentage carboxyl composition used in the MNP modification reaction on the fluorescence intensity observed on the array. The black bars represent the protein attachment reaction to the MNPs with 350 μg of Alexa647–chick IgG, and the gray bars represent the protein reaction procedure to the MNPs with 100 μg of Alexa647–chick IgG. (B) Effect of the EDC method (refer to Table 1) used to attach 100 μg of Alexa647–chick IgG on the surface of 100% carboxyl MNPs. The resulting bar graph shows the relative intensity of the MNPs captured by the array surface for three separate slides. Slide 1 (black) and slide 2 (light gray) involved PBS washes for the MNPs, whereas slide 3 (gray) used Mes washes.

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Table 1
EDC protocols investigated for MNP modification with Alexa647–chick IgG.

EDC exposure method	EDC MNP activation procedure prior to addition of Alexa647–chick IgG
EDC I	EDC/resuspend in IgG solution
EDC II	EDC/magnetic extraction/suspend in IgG solution
EDC III	EDC/magnetic extraction/PBS or Mes wash/magnetic extraction/suspend in IgG solution
EDC IV	EDC + NHS/magnetic extraction/PBS wash/magnetic extraction/suspend in IgG solution
EDC V	EDC ($5\times$)/magnetic extraction/Mes wash/magnetic extraction/suspend in IgG solution

nonuniform array spots, nonreproducible standard deviations, and greater standard deviations in the measured fluorescent intensity signals. To investigate the effect of the EDC method on the amount of Alexa647–chick IgG attached to the surface of the MNPs, five different procedures were performed. These procedures are summarized in Table 1.

The general approach used 100 µl of the prepared Alexa647–chick–MNPs and diluted the sample to 1 ml of PBSCD. Then 0.8 ml of this solution was passed over an Rb–anti-chick IgG-functionalized surface at a flow rate of 0.1 ml/min. The resulting bar graph (Fig. 2B) shows the relative intensity of the Alexa647–chick–MNPs prepared using EDC methods I to V and captured by the rabbit–anti-chick IgG-functionalized surface. The net intensities obtained from the CCD images were normalized to the EDC I procedure, which produced the brightest fluorescence intensity on the slide surface. Slides 1 and 2 used a PBS wash for EDC method III, where these bars are black and light gray, respectively. For slide 3 (dark gray bars), this data set used EDC method III with Mes buffer as the wash solution. The wash procedures attempted to reduce the opportunity for multilayers of proteins to form above the MNP surface and, therefore, reduce aggregation.

As expected, the EDC I procedure resulted in the brightest signals obtained from the Rb–anti-chick IgG-functionalized regions because of a greater chance for multilayer formation. These samples had the greater number of fluorophore-labeled proteins and, therefore, more signaling molecules present on the MNPs. The EDC II procedure included removal of the EDC prior to the addition of Alexa647–chick IgG and resulted in a significant decrease in the overall intensity of the MNPs. This procedure reduced the chance for multilayer formation and, therefore, decreased the number of signaling molecules on the MNP surface. A further reduction in signal was observed in the EDC III procedure, where a PBS wash step was included prior to IgG exposure. The EDC reactive intermediate was found to be more stable using a wash step at the lower pH (Mes [pH 5.0–5.5] vs. PBS [pH 7.4]) and produced a higher relative intensity for the Mes washed samples in Fig. 2B. All variations of the EDC II to V preparation conditions were not tested unless the initial trials either reduced aggregation or produced higher signals than the EDC I method.

Neither the addition of NHS (EDC IV, light gray bar), which is reported to stabilize the reactive EDC intermediate, nor an excess of EDC (EDC V, dark gray bar) seemed to increase the intensity of the signal obtained relative to the EDC III procedure. UV–Vis absorbance of the MNPs in suspension also confirmed that more IgG was removed from the reaction solution when EDC I versus EDC III was used (data not shown). Although EDC method I probably results in multilayers of Alexa647–chick IgG on the surface of the MNPs, it also produces the brightest fluorescence signals when the MNPs are captured on the surface of the biosensor. Therefore, EDC method I along with addition of 100 µg Alexa647–chick IgG to the EDC-activated MNPs that were made in the presence of 100% carboxyl–silane, was used as the optimized MNP protocol,

and PBS/0.1% casein/0.05% DOC was used as the buffer in the immunocapture experiments.

MNP extraction

The next step was to investigate the extraction/concentration procedures, outlined in Scheme 2, of the Alexa647–chick–MNPs to determine how the MNPs perform in a direct assay format and to characterize the impact of the target concentration process on the assay. For this investigation, three distinct sample preparation protocols were used. To begin, 200 µl of the Alexa647–chick–MNPs was diluted in 10 ml of PBSCD. Path A was simply a 1-ml aliquot of the original diluted sample with no extraction/concentration procedure performed on the sample. This sample was used as the reference (Ref) for comparison purposes. Path B provided a control for the extraction process without concentration; a 1-ml aliquot of the original diluted sample was concentrated using a magnet and suspended in a fresh 1-ml aliquot of the PBSCD run buffer. This sample was labeled extracted (Ex). Path C was a 1-ml aliquot of the original diluted sample that underwent the extraction procedure and was then concentrated in a 0.2-ml aliquot of the PBSCD run buffer, resulting in an effective sample concentration of five times that of the original diluted sample. This sample was labeled concentrated (Conc). Path C was performed a total of five times to obtain a 1-ml sample to be used for characterization.

To be efficient, the extraction time required to recover the bulk of the MNPs from the large volume samples was studied. For extraction, an Eppendorf magnet was used to extract the Alexa647–chick–MNPs. The Eppendorf magnet system can hold up to 6×1.5-ml Eppendorf tubes. For the extraction study, four 1-ml aliquots of the diluted sample were placed in the magnet apparatus, and after 3, 7, 15, or 30 min, the sample supernatant was collected. This was achieved by removal of the liquid portion of the sample from the Alexa647–chick–MNPs, and the particle samples were suspended in 0.5 ml of PBSCD. This resulted in a concentration factor of 2. The extracted Alexa647–chick–MNPs and a portion of the preextracted sample (both 0.5 ml) were passed over an antibody-patterned surface at a flow rate of 0.1 ml/min. Relative signal intensity versus extraction time, taken from the CCD image (data not shown), suggested that 15 min was optimal for the extraction procedure. These data also demonstrated that, although the Alexa647–chick–MNPs were concentrated by a factor of 2 as determined by UV–Vis spectroscopy, this did not translate to an increased intensity from the CCD array biosensor image for the concentrated samples. In fact, the fluorescence appeared to be slightly lower than that for the original preextracted sample. Sonication of the concentrated Alexa647–chick–MNPs for 5 min versus the previously used 1-min sonication time gave a signal intensity improvement up to a factor of approximately 2. This suggests that the drop in signal that was observed when the samples were extracted was likely due to aggregation. Aggregation of the Alexa647–chick–MNPs led to the larger particle effect (described previously), which essentially indicated that larger particles were not as effectively captured by the array surface under the shear force of the flow conditions (see Supplementary Fig. 1). Therefore, these results indicate that magnetic extraction can have a detrimental effect on the performance of the MNPs as a tracer in immunoassays. This phenomenon was further investigated to improve the performance of extracted particles on the array system.

A number of extraction experiments using the optimized Alexa647–chick–MNPs coupled with a 5-min sonication prior to use on the immunoassay were performed. Alexa647–chick IgG (100 µg) was attached to the surface of 100% carboxyl-modified MNPs using the EDC I method (no wash). The Alexa647–chick–MNPs (200 µl) were diluted to 10 ml in PBSCD as described above for Scheme 2 using the 15-min extraction time where appropriate.

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Table 2
Average solution and surface measurement characterization of the Alexa647–chick–MNP samples pre- and postextraction displayed as Conc/Ref ratio (see Scheme 2).

Chick–MNP sample	Solution absorbance (400 nm)	Solution fluorescence emission (670 nm)	Surface fluorescence
MNP	3.0	2.3	1.2
MNP overnight	2.9	2.1	1.6
5A:1P PEG A	3.0	1.6	0.9
1A:1P PEG A	4.0	2.4	1.3
1A:5P PEG A	4.5	2.6	1.5
1A:10P PEG A	4.3	1.9	0.6
5A:1P PEG B	2.8	1.4	0.6
1A:1P PEG B	3.6	2.4	1.0
1A:5P PEG B	3.7	2.0	0.9

For the Ref and Conc samples, these samples were sonicated for 5 min, and 50 µl of these samples was diluted in 950 µl of 18 mΩ Milli-Q water. This was done so that UV–Vis and fluorescence spectroscopy measurements could be obtained. Table 2 summarizes the relative ratio of the Conc versus the Ref samples for solution and surface characterization of the NRL Array Biosensor for an average of five separately prepared batches of these Alexa647–chick–MNPs. The concern was that the MNPs were being lost through the extraction procedure. Therefore, UV–Vis spectroscopy was used to determine whether the MNPs themselves were being removed from solution.

The solution UV–Vis values obtained at 400 nm demonstrated that an increase in the concentration of the MNPs in the solution was achieved. Likewise, the solution fluorescence at 650 nm Alexa647–chick–MNPs and the intensity taken from the CCD images from the Rb–anti-chick IgG-functionalized regions following the immunoassay were obtained. As shown in Table 2, both the solution absorbance and, to a lesser extent, the solution fluorescence showed an increase in intensity following extraction. The absorbance result suggests that the MNP samples were effectively collected through the magnetic extraction procedure but that the extraction procedure has an adverse effect on the fluorescence signaling. In addition, the observed concentration did not translate to a significant increase in CCD surface fluorescence signal generated from the immunoassay-captured Alexa647–chick–MNPs. This is likely a result of MNP aggregation and larger particles that might not be as effectively captured by the surface under the flow conditions of the assay. In addition, this observation was also noted for samples prepared using the EDC III method. Therefore, the potential for multilayers of chick IgG on the surface of the MNPs causing these observations can be effectively ruled out. At this point, the MNPs have been demonstrated to function well either as the tracer for the Array Biosensor or as the concentration agent, but when using the MNPs for both functions simultaneously, further investigation into the effect that magnetic extraction has on the MNP array binding event needs to be performed.

The next phase in this investigation was to determine whether the lower than expected increase in CCD fluorescence signal generated from the immunoassay-captured Alexa647–chick–MNPs was a result of the extraction or the concentration of the MNPs. An extra control sample was added to the immunoassay called the Ex sample (see Scheme 2, path B). Although the absorbance measurements suggested that the concentrations of the Alexa647–chick–MNPs in the Ref and Ex solutions were the same (Scheme 2, paths A and B, respectively), both the solution fluorescence and surface intensity taken from the CCD image suggest a drop in the fluorescence for the Alexa647–chick–MNPs following extraction (see the lower half of Fig. 3). This suggested that the extraction process itself was affecting the fluorescence from the MNPs. Aggregation of the Alexa647–chick–MNPs may produce particles too large to remain bound to the surface during the shear force that occurs at
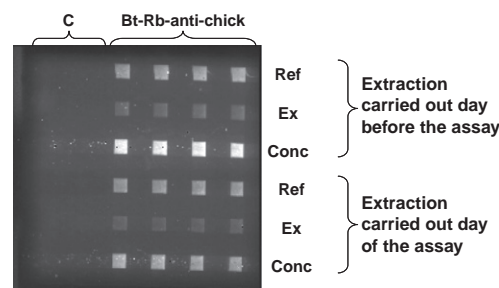

Fig.3. Effect of time of analysis on the assay results. The samples were prepared for the assay as follows: Ref, Ex, and Conc (refer to Scheme 2). This procedure was performed either the day before the assay was used and the samples were stored in PBS at 4 °C (overnight) or on the day of the assay. The samples were sonicated for 5 min prior to use. The resulting CCD image is shown. C, control; Bt, biotinylated.

the surface under the flow conditions of the assay. This may explain the decrease in signal from the surface. It was found that if extracted samples were allowed to sit overnight in the refrigerator before performing the immunoassay, a significant increase (nearly double) in the fluorescent signal obtained from the CCD image of the surface was observed (Fig. 3 and Table 2, MNPs overnight). These data suggested that if MNP aggregation was the cause of these lower signal intensities, the aggregation was at least partially reversible, and further investigation to overcome this problem was performed.

Optimized MNPs for extraction and improved array performance

The main issue with extracting the samples and leaving the MNPs overnight prior to assay is the incompatibility for rapid analysis time. Therefore, we decided to investigate the addition of PEG to the surface of the Alexa647–chick–MNPs to see whether this
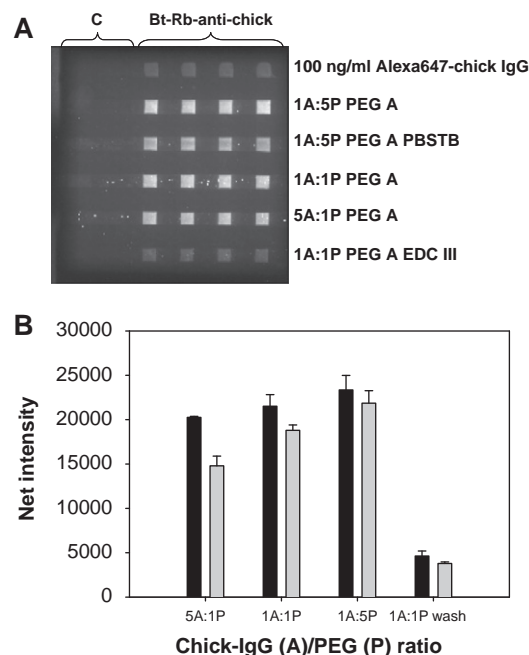

Fig.4. Effect of modification of MNPs with Alexa647–chick IgG and PEG molecules. Alexa647–chick IgG in the presence of either PEG A (5000 MW) or PEG B (10,000 MW) at different mole ratios was attached to the MNPs using the EDC I method (no wash) or the EDC III method (Mes wash). (A) The resulting CCD image for the different MNPs modified with Alexa647–chick IgG/PEG A. Bt, biotinylated. (B) Bar graph plotting the net intensities taken from the CCD image for the different Alexa647–chick IgG MNPs modified with either PEG A (black) or PEG B (gray) molecules.

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would help to prevent the aggregation. PEG is known to prevent nonspecific interactions by increasing the hydrophilic characteristics of the nanoparticles [42]. In this case, the PEG was employed to reduce the particle–particle interactions and the nonspecific interactions occurring between the functionalized nanoparticles and the array surface. Carboxyl-activated MNPs, activated using either the EDC I (no wash) or EDC III (Mes wash) protocol, were simultaneously exposed to Alexa647–chick IgG (100 µg) and either amine–PEG A (5000 MW) or amine–PEG B (10,000 MW) at different mole ratios. Alexa647–chick–PEG–MNPs were then diluted 50 µl in 1 ml of PBS/0.1% casein/0.05% DOC. The samples (0.8 ml) were passed over an antibody-patterned surface at a flow rate of 0.1 ml/min. The resulting CCD image for the different Alexa647–chick IgG/PEG A-modified MNPs is shown in Fig. 4A (PEG B not displayed in image). The bar graph (Fig. 4B) plots the net intensities taken from the CCD image for the different Alexa647–chick–MNPs modified with either PEG A (black) or PEG B (gray) molecules. As illustrated in Fig. 4B, the smaller PEG A-modified Alexa647–chick–MNPs produce slightly stronger fluorescent signals from the CCD image than does the corresponding ratio of PEG B-modified Alexa647–chick–MNPs. For both PEG molecules (A and B), the fluorescence intensity increased slightly with increasing PEG ratios compared with the Ex samples.

Data from the extraction experiments are summarized in Table 2 for both the solution and surface characterization. The relative intensity ratio determined from the intensity of the Conc sample divided by the intensity of the Ref sample is presented. The ratios are compared in the UV–Vis spectroscopy at 400 nm, in the solution fluorescence at 670 nm, and the average fluorescence intensities determined from the CCD array image. The extraction experiments with Alexa647–chick–MNPs modified with the smaller of the amine–PEG molecules (PEG A, 5000 MW) showed promising initial data. The Alexa647–chick–MNPs modified with PEG A, at a ratio of 1:5 chick IgG/PEG A, provided a similar enhancement in CCD fluorescent signal in less time than the regular Alexa647–chick–MNPs extracted and left overnight. Therefore, using PEG A in a commodification procedure with 100 µg of Alexa647–chick IgG on the previously described optimized MNPs improved immunoassay performance with the shortest time between extraction and analysis.

Conclusion

This extensive study has demonstrated the use of MNPs as tracers for immunoassays performed on a biosensor surface and characterized the effect that extraction of the MNPs has on the performance of these MNPs. The use of MNPs for the simultaneous function of target preconcentration and signal transduction in biosensor assays performed under flow conditions was demonstrated using a direct binding assay format. The optimal conditions for synthesizing the MNPs were determined by exploring the surface composition, antibody functionalization procedures, and various blocking buffers. In addition to investigating the MNP synthesis, the best extraction time and method for introducing the concentrated MNPs to the biosensor were ascertained. We determined that magnetic separation of the MNPs had an adverse effect on these modified particles, and further study into nanoparticle surface treatment was performed by adding PEG polymers along with the antibody during the immobilization step.

Previous experiments using micron-sized MPs in conjunction with sensing under flow conditions were found to be problematic. The fabrication of nano-sized MNPs was essential to reduce the shearing effect of the fluid flow on the surface-bound particles. Including the MNPs in the assay instead of removing the bound target or requiring the addition of a secondary fluorescent species minimized the number of steps in the assay and reduced the

chance of losing the target analyte. Furthermore, little time was added to the overall assay protocol, and the target was concentrated prior to performing the analysis. The next step will be to demonstrate sandwich assays where the analyte is pulled out of solution by the MNPs coated with fluorescent antibodies and concentrated prior to biosensor analysis.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ab.2010.11.005.

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